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Infection capacities in the orange-pathogen relationship: Compatible (*Penicillium digitatum*) and incompatible (*Penicillium expansum*) interactions

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ABSTRACT

Penicillium digitatum and *Penicillium expansum* are the most devastating pathogens of citrus and pome fruits, respectively. Whereas *P. digitatum* is a very specific pathogen that only infects *Citrus* fruits, *P. expansum* has a broader host range but has not been reported to be infectious in *Citrus*. To determine the responses of fruits and the infection capacities of both moulds, two varieties of oranges at different maturity stages, different inoculum concentrations and two different storage temperatures were studied. In compatible interactions, no significant differences in rot dynamics among harvests were found with a 10^7 conidia mL^{-1} inoculum concentration at both temperatures tested (20 °C and 4 °C). However, at other inoculum concentrations, significant differences in rot dynamics were found, especially in immature fruits. Incompatible interactions showed that *P. expansum* could infect oranges at commercial maturity in both tested varieties. Decay incidence and severity were higher at 4 °C than at 20 °C. In addition to infection capacity studies, histochemical tests were performed to detect wound-healing compounds for both pathogens. A positive reaction for lignin was detected for both pathogens in immature oranges over a short period (48 h). In all cases, no reactions were found in control samples. Our results indicate that pathogen concentration, host maturity and storage temperature can play important roles in the defence mechanisms of fruit. Furthermore, to our knowledge, this is the first work that demonstrates that *P. expansum* can infect oranges under favourable conditions.

1. Introduction

Penicillium digitatum and *Penicillium expansum* are the most devastating pathogens of citrus and pome fruits, respectively, and are responsible for important economical losses during post-harvest handling. Whereas *P. digitatum* is a very specific pathogen that infects *Citrus* fruits and has not been shown to infect other hosts, *P. expansum* has a broad host range. To our knowledge, *P. expansum* has not been shown to cause post-harvest disease on *Citrus* fruits; thus, it is considered a non-host pathogen or an incompatible interaction. Currently, the use of synthetic fungicides constitutes the main method to control these post-harvest diseases; however, the use of chemicals is becoming increasingly restricted because of concerns about environment and health, as well as the development of fungicidal resistance in pathogens (Viñas et al., 1993). In spite of the application of fungicides and the increased implementation of new alternative strategies, green mould in *Citrus* fruits and blue mould in pome fruits continue to place high infection pressures on stored fruits worldwide. These facts justify the need and the interest for more detailed studies on host–pathogen interactions to increase our knowledge of both pathogen virulence mechanisms and host defence mechanisms. This will serve as an initial step leading to the rational design of new and safer control strategies.

In an incompatible interaction, the avirulent pathogen is recognised via the action of disease resistance (R) gene products, eliciting an accumulation of biphasic reactive oxygen species (ROS) with a low-amplitude, transient first phase, followed by a sustained phase of much higher magnitude that correlates with disease resistance (Lamb and Dixon, 1997). When this recognition occurs, the pathogen cannot infect the plant. In contrast, in a compatible interaction, virulent pathogens avoid host recognition, inducing only the transient, low-amplitude first phase of this response. The lack of the second phase is thought to play an important signalling role in the activation of plant defences. In fact, the important ROS accumulation during the second phase has been reported to

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precede the hypersensitive response that often occurs during pathogen recognition, leading to an incompatible interaction (Levine et al., 1994). In spite of the important role of H_2O_2 in plant defence (Borden and Higgins, 2002; Li et al., 2005), there are few reports of the role that it might play in fruit; it has been described on apples (Castoria et al., 2003; Torres et al., 2003), mangoes (Zeng et al., 2006) and strawberries (Brown et al., 2008). Regarding citrus fruits, only lemons (Macarisin et al., 2007) and oranges (Torres et al., 2011) have been analysed to characterise the potential role of H_2O_2 during compatible and incompatible interactions.

Different functions have been postulated for ROS production in response to pathogens (Torres, 2010). One function could be to contribute to the establishment of physical barriers at the sites of infection via oxidative cross-linking of the precursor during the localised biosynthesis of lignin and suberin polymers (Huckelhoven, 2007). Early histological investigations showed that the development of resistance to infection is associated with the deposition of a material that turns red in the presence of phloroglucinol-HCl (PG-HCl) in tissues adjacent to the injuries (Baudoin and Eckert, 1985; Brown and Barmore, 1983). However, the nature of this material was not clear because some authors described it as lignin and wound gum (Baudoin and Eckert, 1985; Brown and Barmore, 1983; Stange et al., 1993). In a more recent study, cured grapefruits stored at 33 °C for 48 h excluded lignin as a component in the newly formed material, and NMR spectroscopy provided further evidence that the induced material was suberin (Lai et al., 2003).

The differences in the final outcome of a plant–pathogen interaction, either susceptibility or resistance, might be due to the timing and intensity of the plant's defence responses (Tao et al., 2003). Thus, the maturity stages of fruits at harvest could be among the main factors determining the susceptibility of fruits to mechanical damage or infection during post-harvest storage (Davey et al., 2007; Torres et al., 2003). Ambient conditions may also play an important role in wound healing and resistance to infection in citrus fruits (Brown, 1975). Until now, post-harvest research has mainly focussed on different ways to control moulds (Janisiewicz and Korsten, 2002; Tripathi and Dubey, 2004; Wilson et al., 1991), while little is known about the effects of fruit maturity on mould growth.

The aim of this study was to investigate the infection capacities of the pathogens *P. digitatum* (compatible) and *P. expansum* (incompatible) in two varieties of oranges (Navelina and Valencia) at different (i) maturity stages; (ii) pathogen inoculum concentrations; and (iii) storage temperatures. A histochemical study was carried out to detect the accumulation of different compounds to define their roles in host resistance against both studied pathogens.

2. Materials and methods

2.1. Fruits

Navelina and Valencia oranges were obtained at different maturity stages from October 2008 to January 2009 (eight harvests ranging from immature to over-matured) and from March 2009 to June 2009 (seven harvests ranging from immature to over-matured), respectively, from a commercial orchard in Tortosa (Catalonia, Spain). For Navelina oranges, harvests one and two were considered as prior to commercial maturity (immature fruit), harvests three to six were considered as commercial maturity (mature fruit), and harvests seven and eight were considered as over-maturity (over-matured fruit). For Valencia oranges, harvests one and two were considered as prior to commercial maturity (immature fruit), harvests three to five were considered as commercial maturity (mature fruit), and harvests six and seven were considered as over-maturity (over-matured fruit). Oranges were used just after harvest.

2.2. Fungal cultures

P. digitatum PDM-1 and *P. expansum* CMP-1 are the most aggressive isolates from our collection capable of infecting citrus and pome fruits, respectively. They are maintained on potato dextrose agar medium (PDA; 200 mL boiled potato extract, 20 g dextrose, 20 g agar and 800 mL water) and periodically grown on wounded citrus (*P. digitatum*) or pome fruits (*P. expansum*) and then re-isolated to maintain virulence. Conidial suspensions were prepared by adding 10 mL of sterile water with 0.01% (w/v) Tween-80 over the surface of 7- to 10-day-old cultures grown on PDA and rubbing the surface of the agar with a sterile glass rod. Cells were counted in a haemocytometer and diluted to different concentrations (10^7, 10^6, 10^5 or 10^4 conidia mL^{-1}) and were then used in each infective capacity study.

2.3. Infective capacity studies

The effects of the maturity stages of oranges, inoculum concentrations and storage temperatures were assessed for both the compatible interaction (*P. digitatum*-oranges) and the incompatible interaction (*P. expansum*-oranges).

Oranges were washed thoroughly with tap water and allowed to dry before artificial inoculation. Oranges were wounded with a nail (1 mm wide, 5 mm long and 2 mm deep) and inoculated with 15 μL aqueous conidia suspensions of pathogen at four different concentrations; 10^7 and 10^6 conidia mL^{-1} are considered in this work as high inoculum concentrations, and 10^5 and 10^4 conidia mL^{-1} are considered as low inoculum concentrations. This methodology was performed individually for each pathogen. The infective capacities of each pathogen were assessed at two different storage temperatures (4 °C and 20 °C) and 85% relative humidity. As soon as visible growth started, the diameter of rot was measured along the time to obtain the development of rot dynamics for each pathogen, inoculum concentration, temperature and maturity stage. Five oranges constituted a single replicate, and each treatment was repeated four times. The experiments were performed with both orange varieties: Navelina (eight harvests) and Valencia (seven harvests).

2.4. Determination of quality parameters

Colour development, loss of firmness, soluble solids and acidity were determined to evaluate the effects of different harvest dates on fruit quality.

Colour was measured on two opposite sides of each fruit using a tri-stimulus colourimeter (Chromameter CR-200, Minolta, Japan). The mean values for the lightness (L*), red-greenness (a*) and yellow-blueness (b*) parameters were calculated for each fruit and expressed as Colour index (CI) = (1000*a)/(L*b). Firmness measurements were performed using a TA-XT2i Texture Analyser (Stable Micro Systems Ltd., Surrey, UK), based on the millimetres of fruit deformation resulting from fruit responses to 2 kg of pressure on the longitudinal axis at a constant speed of 2 mm s^{-1}. Total soluble solids content (TSS) and titratable acidity (TA) were assessed in juice using a refractometer (Atago, Tokyo, Japan) and by titration of 10 mL of juice with 0.1 N NaOH and 1% phenolphthalein as an indicator. Data on maturity indexes represent the means of 20 individual fruits. Maturity index was calculated as a ratio of TSS/TA.

2.5. Histochemical tests

The development of resistance was studied by wounding Valencia oranges at three maturity stages: immature (harvest one), commercial (harvest four) and over-matured (harvest seven). Oranges were inoculated with *P. digitatum* or *P. expansum* at 10^7 or 10^4 conidia mL^{-1} concentrations. Control fruits were wounded but not inoculated. Fruits were stored at 20 °C and 85% RH for 0, 24, 48 and 72 h and 7 d.

After each time, excised peel (flavedo and albedo) tissue cylinders (5 mm inside diameter and 4 mm deep) containing wounds were infiltrated with FAA (formalin, glacial acetic acid, 96% ethanol and water 10:5:50:35 v/v) and fixed for no more than 48 h. Cylinders were dehydrated in an ethanol-xylene series, embedded in paraffin, sectioned transverse at a thickness of 20 μm with a rotator microtome and fixed to glass slides with Haupt adhesive and heat. Sections were deparaffinised with xylene and brought to miscibility with water to apply the following histochemical tests:

I. A Maüle reaction for lignin was performed according to the method described by Thomson et al. (1995) with slight modifications. Sections on slides were stained with 1% (v/v) aqueous potassium permanganate for 15 min, rinsed three times with distilled water (30 s each rinse), placed in 1% (v/v) HCl for 4 min, rinsed in water and then placed in 0.025% (v/v) ammonia for 1 min. The sections were rinsed in distilled water for 1 min, followed by 70% ethanol for 2 min. The sections were mounted in glycerine.
II. A toluidine blue O test for lignin was performed according to the method described by Krishnamurthy (1999). Sections on slides were stained in aqueous toluidine blue O solution, pH 4.4 (0.05% stain in benzoate buffer [0.25 g benzoic acid and 0.29 g sodium benzoate in 200 mL water]). They were then washed and mounted in distilled water.
III. A Sudan IV test for suberin was performed according to the method from Johansen (1940) with slight modifications. Sections on slides were immersed in Sudan IV solution for 10 min. The Sudan IV solution was prepared by adding 50 mL of glycerine to 50 mL of a saturated solution of Sudan IV in 95% ethanol and filtering. Sections were rinsed in 70% ethanol and then mounted in glycerine.
IV. A lacmoid test for callose was performed according to the method described by Krishnamurthy (1999) with slight modifications. Sections on slides were stained in 0.25% (w/v) solution of lacmoid in 30% ethanol and rinsed with 1% sodium bicarbonate in 50% ethanol for a few seconds. The sections were rinsed in 70% ethanol and then mounted in glycerine.

Samples were analysed with both a Leica MZ16F stereoscope and a Leica DM5000 microscope. Images were acquired using a Leica colour digital camera (Leica DFC 420).

In this work, samples at 0 or 24 h after inoculation did not show reactions for control, *P. digitatum* or *P. expansum* inocula with any of the stains used. Oranges infected with *P. digitatum* showed complete rot development after 72 h of incubation, and histochemistry was not performed at that time. Therefore, 48 h after inoculation was considered a short-period response because at this time, samples could be excised (with the exception of mature and over-matured oranges inoculated with 10^7 conidia mL^{-1} *P. digitatum*) and showed a stain reaction. Seven days after inoculation was considered a long-period response to *P. expansum*.

2.6. Data analysis

Data regarding the growth rates of decayed fruit, visible initial rotting day and quality parameters were analysed for significant differences by analysis of variance (ANOVA) with the statistical package SAS (Microsoft). Statistical significance was deemed when $P < 0.05$; when the analysis was statistically significant, a Student-Newman-Keuls (SNK) test for separation of means was performed.

For the growth studies, the radial growth rate (cm day^{-1}), inoculum concentration and temperature for each harvest were obtained from the growth data using linear regression of the linear parts of the temporal growth curves.

3. Results

3.1. Effect of maturity stage and inoculum concentration on the compatible interaction at 20 °C

The results obtained in Valencia oranges infected with *P. digitatum* at different inoculum concentrations and incubated at 20 °C are shown in Fig. 1. For all harvest dates, the growth for this pathogen followed a linear pattern at high inoculum concentrations (Fig. 1A and B). In contrast, low inoculum concentrations showed an exponential growth pattern (Fig. 1C and D) that was more pronounced with oranges harvested early (harvest one in particular).

In general, only harvest one (the greenest fruits tested) showed a different growth pattern than the other harvests, and consequently, there were no differences between commercially mature and over-matured harvest behaviours (Fig. 1). Differences between the more immature fruits and the rest of the harvests were more pronounced at decreased inoculum concentrations. After 6 d of inoculation at 10^7 and 10^6 conidia mL^{-1}, lesion diameter averages were approximately 12 and 10.5 cm, respectively. Meanwhile, at lower inoculum concentrations, rot diameter averages were only approximately 9 and 5 cm, respectively.

Statistical analysis revealed that growth rate was not different between inoculum concentrations at any harvest dates (data not shown); meanwhile, statistical differences were observed for the visible initial rotting day. The first visible rot symptoms (Table 1) appeared earlier at high concentrations (10^7 conidia mL^{-1} – 2 days) than at low inoculum concentrations (10^4 conidia mL^{-1} – 2–4.5 days). However, for over-matured harvests, no significant differences were found in visible initial rotting day between inoculum concentrations.

When analysing inoculum concentrations (Table 1), at 10^7 conidia mL^{-1}, no significant differences among harvests for growth rate or visible initial rotting day were found. For growth rate, at 10^6 conidia mL^{-1}, only harvest one (the greenest harvest) showed significant differences between commercially mature and over-matured harvests; at 10^5 conidia mL^{-1}, immature harvests showed significant differences compared to over-matured harvests; and at 10^4 conidia mL^{-1}, harvest one showed significant differences compared to the rest of harvests. For visible initial rotting day, at 10^6 conidia mL^{-1}, no significant differences between harvests were found; at 10^5 conidia mL^{-1}, only over-matured harvests were statistically different from the other harvests; and at 10^4 conidia mL^{-1}, four different groups had statistically significant differences.

Similar patterns and tendencies were obtained for Navelina oranges (data not shown).

3.2. Effect of maturity stage and inoculum concentration on the compatible interaction at 4 °C

Lesion diameters of Valencia oranges inoculated with *P. digitatum* at different inoculum concentrations and incubated at 4 °C are shown in Fig. 2. At all inoculum concentrations, rot dynamics always displayed an exponential pattern.

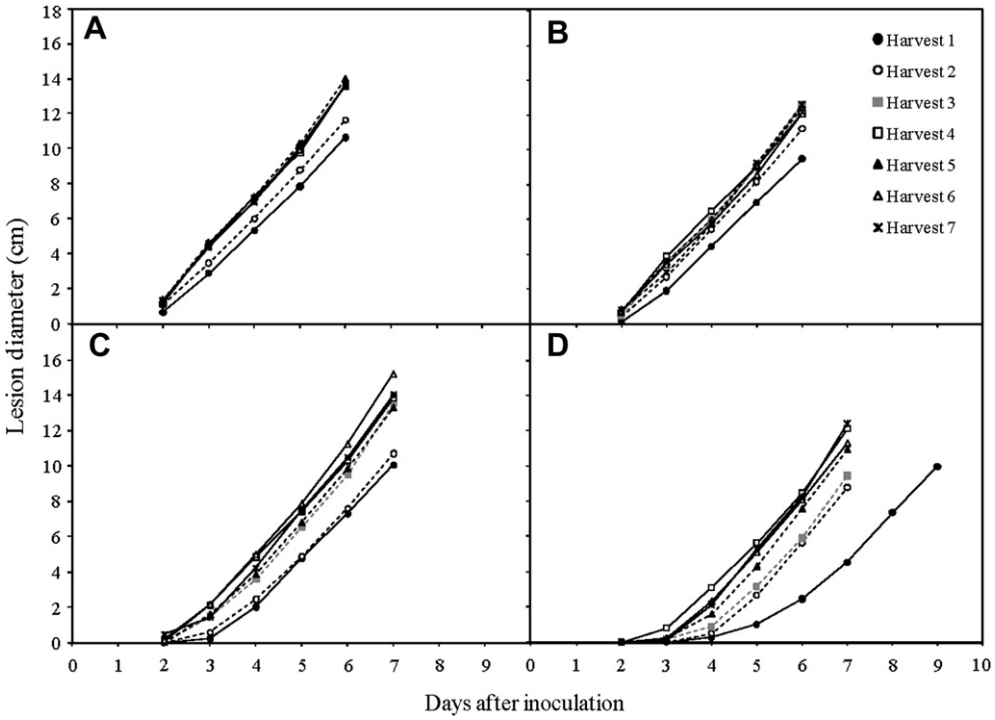

Fig. 1. Influence of maturity stage on lesion diameter (cm) in the compatible interaction at 20 °C and 85% RH. Valencia oranges were harvested at seven different dates and inoculated with *Penicillium digitatum* at four different inoculum concentrations: 10^7 conidia mL^{-1} (A), 10^6 conidia mL^{-1} (B), 10^5 conidia mL^{-1} (C) and 10^4 conidia mL^{-1} (D). Each point represents the mean of 20 fruits.

In general, at high inoculum concentrations, all harvests had similar growth patterns (Fig. 2). However, at low inoculum concentrations, immature harvests showed a different growth pattern than the other harvests. Differences between immature harvests and the other harvests were more pronounced at decreased inoculum concentrations. After 40 d of incubation, lesion diameter averages for immature harvests at 10^5 and 10^4 conidia mL^{-1} were approximately 4–5 cm and 2–4 cm; for the rest of the harvests, lesion diameters were >8 cm and 7 cm, respectively.

Table 1
Growth rates and visible initial rotting days of *Penicillium digitatum* in Valencia oranges at four different inoculum concentrations, seven different harvests and two different temperatures. For each inoculum concentration, harvests with different letters are statistically different according to the SNK test ($P < 0.05$).

Inoculum concentration	Harvest	20 °C		4 °C	
		Growth rate (cm d^{-1})	Visible initial rotting day (d)	Growth rate (cm d^{-1})	Visible initial rotting day (d)
10^7	1	2.49 a	2.0 a	0.52 a	14.0 a
	2	2.59 a	2.0 a	0.52 a	11.0 b
	3	2.88 a	2.0 a	0.52 a	10.0 c
	4	2.86 a	2.0 a	0.53 a	10.5 cb
	5	2.94 a	2.0 a	0.54 a	10.0 c
	6	2.89 a	2.0 a	0.54 a	7.0 d
	7	2.91 a	2.0 a	0.57 a	10.0 c
10^6	1	2.53 b	2.3 a	0.46 d	17.0 a
	2	2.70 ab	2.3 a	0.46 d	15.0 b
	3	2.87 a	2.0 a	0.49 cd	14.0 cb
	4	2.81 a	2.0 a	0.51 bc	13.0 c
	5	2.98 a	2.0 a	0.52 bc	14.0 cb
	6	2.88 a	2.0 a	0.53 b	13.0 c
	7	3.00 a	2.0 a	0.57 a	14.0 cb
10^5	1	2.50 b	3.0 a	0.42 c	26.0 a
	2	2.54 b	3.0 a	0.46 bc	18.0 b
	3	2.76 ab	3.0 a	0.50 abc	17.0 b
	4	2.80 ab	3.3 a	0.53 ab	17.5 b
	5	2.86 ab	3.0 a	0.53 ab	17.0 b
	6	3.11 a	2.3 b	0.54 ab	14.0 c
	7	3.15 a	2.0 b	0.57 a	17.0 b
10^4	1	2.31 b	4.5 a	0.42 b	29.5 a
	2	2.78 a	4.0 b	0.47 ab	22.8 b
	3	2.84 a	3.0 c	0.55 a	18.0 c
	4	2.99 a	3.0 c	0.56 a	19.0 c
	5	3.13 a	3.0 c	0.57 a	18.0 c
	6	3.00 a	2.3 d	0.56 a	19.0 c
	7	3.21 a	2.0 d	0.58 a	19.0 c

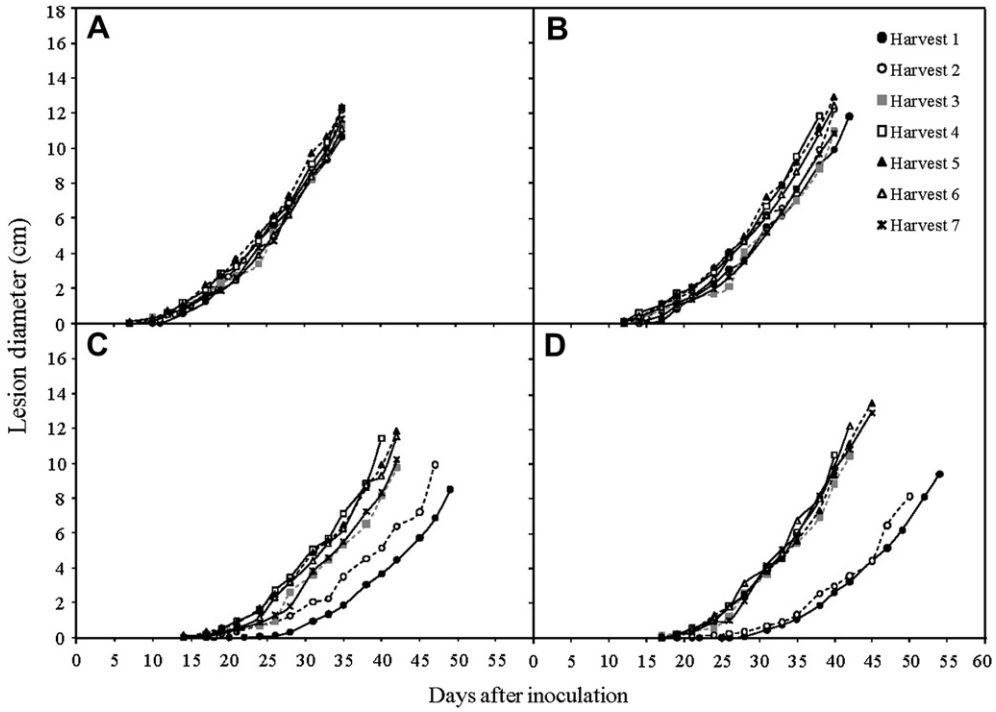

Fig. 2. Influence of maturity stage on lesion diameter (cm) in the compatible interaction at 4 °C and 85% RH. Valencia oranges were harvested at seven different dates and inoculated with *Penicillium digitatum* at four different inoculum concentrations: 10^7 conidia mL^{-1} (A), 10^6 conidia mL^{-1} (B), 10^5 conidia mL^{-1} (C) and 10^4 conidia mL^{-1} (D). Each point represents the mean of 20 fruits.

The results obtained for the effects of inoculum concentration on growth rate and visible initial rotting day were similar to those obtained at 20 °C for all maturity stages. The growth rates were not statistically different between inoculum concentrations for any harvest dates (data not shown); however, visible initial rotting day increased significantly at decreased inoculum concentrations. The first visible symptoms of decay (Table 1) appeared earlier at high inoculum concentrations (10^7 conidia mL^{-1} – 7–14 days) than at low inoculum concentrations (10^4 conidia mL^{-1} – 18–29.5 days). Moreover, significant differences were found between inoculum concentrations for each harvest date.

Table 1 shows the statistical analysis for growth rate and visible initial rotting day at different harvests and for each inoculum concentration. For growth rate, at 10^7 conidia mL^{-1}, no significant differences between harvests were found. However, the most important differences between harvests were found for an inoculation dose of 10^4 conidia mL^{-1}: harvest one showed significant differences between commercially mature and over-matured harvests. The differences in the first visible symptoms of decay (Table 1) were more pronounced between harvests at lower inoculum concentrations.

Navelina oranges showed similar exponential patterns of rot dynamics and tendencies in rot development for all harvests (data not shown).

3.3. Effect of maturity stage and inoculum concentration on the incompatible interaction at 20 °C

Rot lesion diameter at 20 °C storage temperature was monitored to evaluate the effects of maturity stage and pathogen concentration (Fig. 3).

Depending on the combination of factors (maturity stage and inoculum concentration), the *P. expansum*-oranges interaction can change from incompatible to compatible in both Valencia and Navelina varieties. When *P. expansum* was not able to infect oranges, visible changes in flavedo (an orange-red-coloured circle around inoculated wounds) and albedo (death tissue) were observed (Fig. 4). These reactions showed a concentration-dependent behaviour; the biggest reaction was observed at a 10^7 conidia mL^{-1} inoculum concentration. Moreover, at low inoculum concentrations, *P. expansum* was not able to develop infection regardless of maturity stage or orange variety.

Results obtained in Valencia oranges showed that *P. expansum* was able to infect and develop rot in the commercially mature harvest at 10^7 conidia mL^{-1} (Fig. 3A) and in over-matured fruits at 10^6 conidia mL^{-1} (Fig. 3B). Lesion diameters observed at 10^7 conidia mL^{-1} inoculum concentration were bigger than the ones observed at 10^6 conidia mL^{-1}. After 17 d, incubation lesion diameter averages were approximately 0.8 cm and 0.3 cm at 10^7 and 10^6 conidia mL^{-1}, respectively.

For Valencia oranges, statistical analysis showed that growth rate and visible initial rotting day were different between inoculum concentrations for the over-matured harvest (data not shown). The first visible symptoms (Table 2) appeared earlier at the 10^7 conidia mL^{-1} inoculum concentration (3 days) than at the 10^6 conidia mL^{-1} inoculum concentration (6.7–8.3 days). No significant differences in growth rate or visible initial rotting day were found between harvests at both concentrations that develop rot (Table 2).

In Navelina oranges, *P. expansum* was able to develop rot in harvest six when the inoculum concentration was the highest (Fig. 3C) and in over-matured oranges at the 10^6 conidia mL^{-1} concentration (Fig. 3D). In general, Navelina oranges presented larger lesion diameters than Valencia oranges. After 11 d of inoculation at 10^7 and 10^6 conidia mL^{-1}, lesion diameter averages were between 1–3.5 and 1–3 cm, respectively.

No differences were found in growth rates between inoculum concentrations (data not shown); meanwhile, statistical differences were observed for visible initial rotting day in harvest eight. In

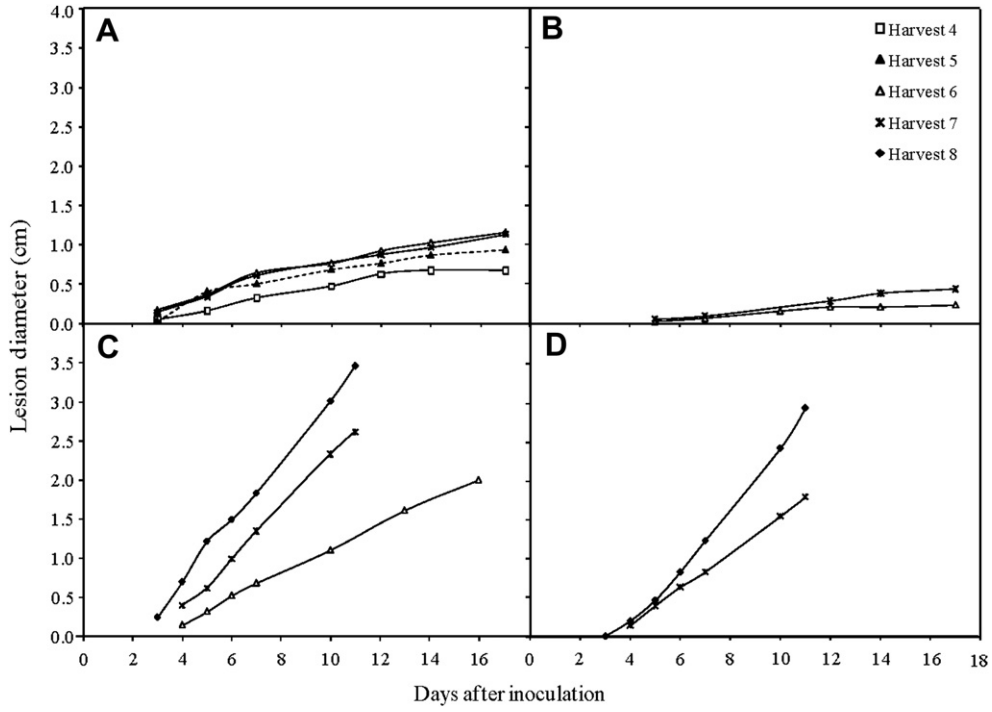

Fig. 3. Influence of maturity stage on lesion diameter (cm) in the incompatible interaction at 20 °C and 85% RH. Valencia (A and B) and Navelina (C and D) oranges were harvested at different maturity stages and inoculated with *Penicillium expansum* at different inoculum concentrations: 10^7 conidia mL^{-1} (A and C) and 10^6 conidia mL^{-1} (B and D). The 10^5 and 10^4 conidia mL^{-1} concentrations did not show rot development in any tested condition or harvest. Each point represents the mean of 20 fruits.

this harvest, visible symptoms of rot appeared earlier at the 10^7 conidia mL^{-1} inoculum concentration (3 days) than at the 10^6 conidia mL^{-1} inoculum concentration (4.2 days).

In relation to inoculum concentration (Table 2), significant differences in growth rate were found between harvests at the 10^7 conidia mL^{-1} inoculum concentration; over-matured oranges showed higher growth rates than harvest six. Nevertheless, visible initial rotting day showed significant differences among harvest eight and the other harvests. At the 10^6 conidia mL^{-1} concentration, the same patterns as those found in Valencia oranges were found, with no differences between harvests for either growth rate or visible initial rotting day.

3.4. Effect of maturity stage and inoculum concentration on the incompatible interaction at 4 °C

For both orange varieties, *P. expansum* could grow at 4 °C only at high inoculum concentrations by following the pattern shown in Fig. 5. However, at low inoculum concentrations, a small number of over-matured oranges showed infection in two varieties assessed (data not shown).

In Valencia oranges, all harvests that were infected with *P. expansum* at 20 °C also showed rot at 4 °C (Fig. 5A and B). Lesion diameters observed with the 10^7 and 10^6 conidia mL^{-1} inoculum concentrations showed similar values and achieved approximately 4.5-cm lesions by 75 days after inoculation.

Statistical analysis showed that no significant differences were found in growth rate between inoculum concentrations (data not shown). However, the visible initial rotting day appeared earlier at 10^7 conidia mL^{-1} (21–28 days) than at 10^6 conidia mL^{-1} (26.3–31.5 days) (Table 2). At the 10^7 conidia mL^{-1} inoculum concentration, harvest four showed significant differences compared to the other harvests; at 10^6 conidia mL^{-1}, no differences were found (Table 2).

In Navelina oranges, all harvests that were infected with *P. expansum* at 20 °C also showed rot at 4 °C, except harvest seven at 10^6 conidia mL^{-1}. At the 10^7 conidia mL^{-1} inoculum concentration (Fig. 5C), two significant groups were observed: over-matured harvests showed higher lesion diameters (around 10 cm) after 70 days than harvest six (around 7 cm). At 10^6 conidia mL^{-1} (Fig. 5D), only harvest eight showed *P. expansum* decay after 19 d of storage conditions. However, at 40 d after inoculation, *Penicillium italicum* contamination was observed in wounds.

Statistical analysis demonstrated that the growth rate at the 10^7 conidia mL^{-1} inoculum concentration was higher than that obtained at 10^6 conidia mL^{-1} for harvest eight (data not shown); meanwhile, no significant differences were found between visible initial rotting days.

Comparing inoculum concentrations (Table 2), the growth rate was lower at a dose of 10^7 conidia mL^{-1} in harvest six than in the other harvests; however, no differences were found between harvests regarding visible initial rotting day.

In general, *P. expansum* lesion diameters were larger in cold conditions than at 20 °C. At the end of the study (75 days at 4 °C and 11 days at 20 °C), lesion diameter averages were around 7–10 and 1–3.5, respectively, for Navelinas at the 10^7 conidia mL^{-1} inoculum concentration. It is also interesting to note the differences in percentages of infected wounds by *P. expansum* between storage temperatures (Fig. 6). At 4 °C, 100% of wounds inoculated with *P. expansum* from harvests six, seven and eight developed infection; meanwhile, at 20 °C, only over-matured harvests achieved this degree of infection.

3.5. Changes in quality parameters

Significant differences in Valencia quality parameters were found between harvest dates (Table 3). The maturity stages of the oranges did not exhibit significant differences in total soluble solids

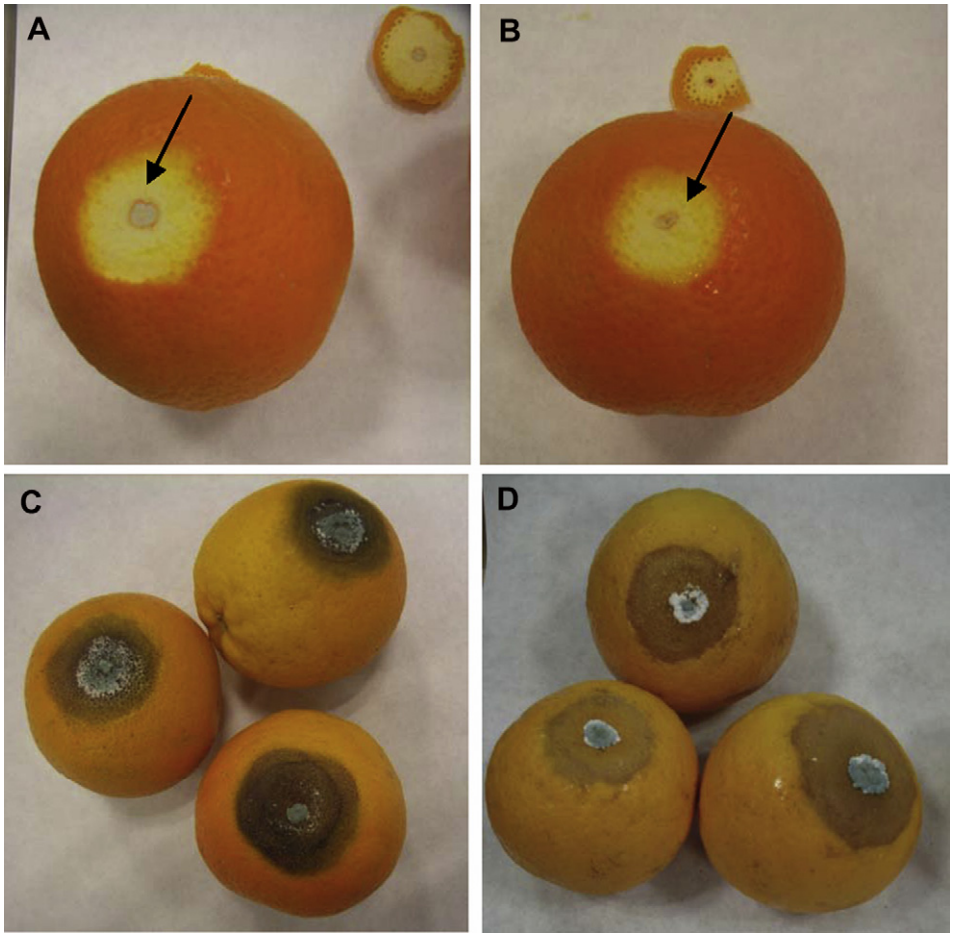

Fig. 4. Valencia oranges inoculated with *P. expansum*. A visible reaction around the inoculation site was found in immature oranges (A) at the 10^7 conidia mL^{-1} inoculum concentration and (B) at the 10^4 conidia mL^{-1} inoculum concentration. In over-matured fruits, *P. expansum* was able to develop an infection with the 10^7 conidia mL^{-1} inoculum concentration at both (C) 20 °C and (D) 4 °C.

(TSS). In contrast, titratable acidity (TA) decreased as the commercial harvest date increased. Accordingly, TSS/TA became higher, and its pattern of change followed a TA pattern. Colour and firmness parameters were not useful to define maturity stages (data not shown).

In Navelina oranges, the changes in quality parameters were similar to those for Valencia oranges, although TSS levels showed slightly lower values in the first harvests (data not shown).

3.6. Histochemical results

The Maüle test resulted in a typical orange-reddish-brown staining in the epicarp cells, which is a positive sign for the presence of lignin compounds. No positive reaction was found around the wound in control samples over either short- (data not shown) or long-period responses (Fig. 7A–C) at the three maturity stages assessed. In oranges inoculated with *P. digitatum* at 10^4 conidia

Table 2
Growth rates and visible initial rotting days of *Penicillium expansum* in Valencia and Navelina oranges at two different inoculum concentrations, five different harvests and two different temperatures. When *P. expansum* was not able to grow, data are not shown. For each inoculum concentration, harvests with different letters are statistically different according to the SNK test ($P < 0.05$).

Variety	Inoculum concentration	Harvest	20 °C		4 °C	
			Growth rate (cm d^{-1})	Visible initial rotting day (d)	Growth rate (cm d^{-1})	Visible initial rotting day (d)
Valencia	10^7	4	0.045 a	4.0 a	0.067 b	28.0 a
		5	0.046 a	4.5 a	0.081 a	21.0 b
		6	0.063 a	3.0 a	0.088 a	22.7 b
		7	0.062 a	3.0 a	0.085 a	21.0 b
	10^6	6	0.012 a	8.3 a	0.082 a	26.3 a
		7	0.034 a	6.7 a	0.084 a	28.0 a
Navelina	10^7	6	0.157 b	4.2 a	0.114 b	19.0 a
		7	0.327 a	4.0 a	0.166 a	19.0 a
		8	0.392 a	3.0 b	0.162 a	19.0 a
	10^6	7	0.234 a	4.2 a	No growth	No growth
		8	0.396 a	4.2 a	0.082	19.0

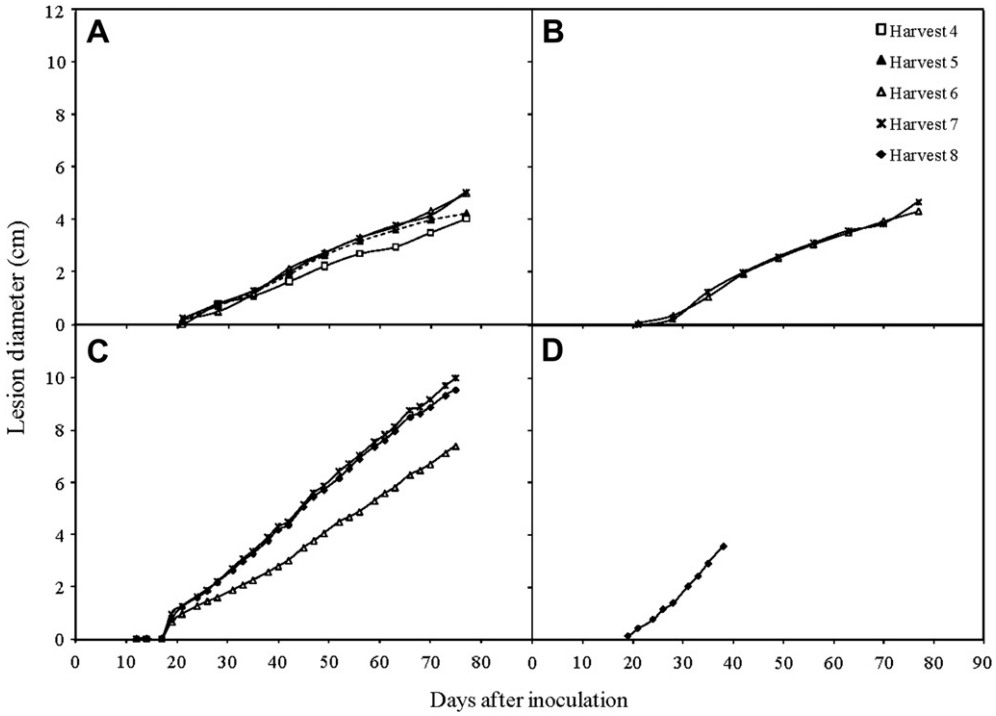

Fig. 5. Influence of maturity stage on lesion diameter (cm) in the incompatible interaction at 4 °C and 85% RH. Valencia (A and B) and Navelina (C and D) oranges were harvested at different maturity stages and inoculated with *Penicillium expansum* at two different inoculum concentrations: 10^7 conidia mL^{-1} (A and C) and 10^6 conidia mL^{-1} (B and D). Each point represents the mean of 20 fruits.

mL^{-1}, no positive reaction was found in any of the three maturity stages (data not shown).

For oranges inoculated with *P. digitatum* at 10^7 conidia mL^{-1}, the Maüle reaction was positive for immature harvests at the short-period response (48 h after inoculation); moreover, signs of rot development were evident by microscopy (data not shown).

At immature harvest and at the short-period response, the Maüle reaction was positive for *P. expansum* inoculated at 10^7 and 10^4 conidia mL^{-1}, and the reaction intensity was correlated with the pathogen concentration (data not shown). In general, the Maüle reaction was of low intensity in the short-period response to *P. expansum* as maturity advanced. In contrast to *P. digitatum* samples (which rotted), wounds inoculated with *P. expansum* in the long-period response could be analysed (7 d after inoculation). The Maüle test showed higher intensity around inoculated wounds in immature and mature fruits (Fig. 7G and H) than in over-matured fruits (Fig. 7I). In the over-matured harvests, oranges inoculated with *P. expansum* at the 10^7 conidia mL^{-1} concentration showed a low-intensity reaction, germinating spores in albedo tissue were found, and the tissue around them was visibly disintegrated (Fig. 7I). Samples infected with *P. expansum* at 10^4 conidia mL^{-1} (Fig. 7D–F) showed a positive reaction but lower intensity than at 10^7 conidia mL^{-1} (Fig. 7G–I).

A positive reaction for lignin was also obtained with a toluidine blue O test (data not shown), and the results were similar to the Maüle reaction (which assessed intensity of reaction and when it appeared). This stain also reveals a brown colour when rot development appears. With this stain, we visualised *P. expansum* germinating spores at 10^4 conidia mL^{-1} in the over-matured harvest; however, rot development was not visually observed in oranges.

Sudan IV and lacmoid reagents for the detection of suberin and callose, respectively, did not show positive reactions in these histochemical assays for any samples studied (control, *P. digitatum* and *P. expansum*; data not shown).

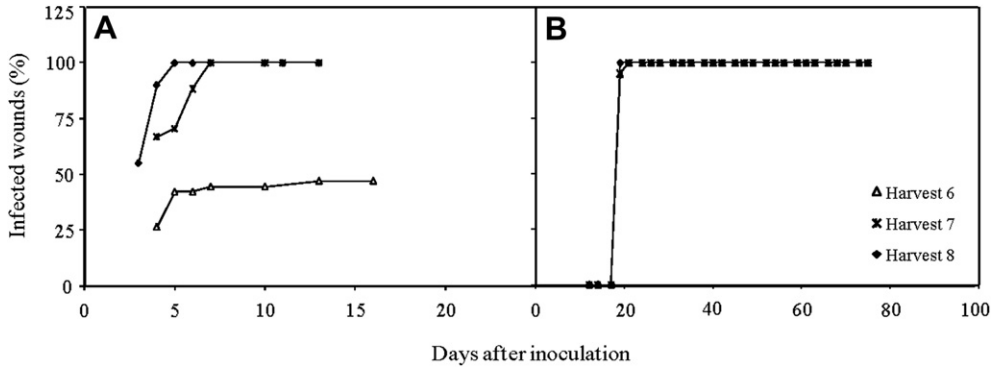

Fig. 6. Effect of storage temperature: 20 °C (A) and 4 °C (B) on the decay incidence caused by *Penicillium expansum* (10^7 conidia mL^{-1}) on Navelina oranges from different maturity harvests. Each point represents the mean of 20 fruits.

Table 3
Effect of harvest date on soluble solids, citric acid content and the ratio of TSS/TA on Valencia oranges. Harvest dates with the same letter are not statistically different ($P < 0.05$) according to the SNK test.

Harvest	Date	Total soluble solids (TSS in %) *	Titratable Acidity (TA in g l^{-1} citric acid)	Ratio TSS/TA
1	20/03/09	10.4	1.22 b	8.5 d
2	03/04/09	10.9	1.37 a	7.9 d
3	24/04/09	10.9	1.24 b	8.8 d
4	08/05/09	10.9	1.02 c	10.7 c
5	22/05/09	10.9	0.92 d	11.8 b
6	05/06/09	11.2	0.87 d	12.9 b
7	19/06/09	11.1	0.73 e	15.2 a

* Indicates no significant differences.

4. Discussion

In this work, the capacities of *P. digitatum* and *P. expansum* to infect two varieties of oranges at different maturity stages and storage temperatures were assessed. The inoculation of a compatible pathogen (*P. digitatum*) at different inoculum concentrations always showed the development of rot. In contrast, the non-host (incompatible) pathogen, *P. expansum*, was only able to infect oranges under specific conditions (mature and over-matured fruit) and was dependent on storage temperature and inoculum concentration.

P. digitatum specificity to citrus fruit is well known (Adams and Moss, 2000). This study has shown that no alterations of the assessed factors could prevent the development of this pathogen in oranges, thus confirming the virulence and specificity of this pathogen under a wide range of favourable and unfavourable conditions. Maturity stage appears to be an important factor in determining the resistance of oranges to *P. digitatum* regardless of the orange variety used, although, in general, Navelina oranges demonstrated more sensitivity to infection than Valencia oranges.

Our results indicated that immature oranges stored at 20 °C showed significant differences in rot dynamics, with slower growth rate development in relation to over-matured harvests. The exception to this is at the 10^7 conidia mL^{-1} inoculum concentration, at which no differences were found between harvest dates. These results differ from those obtained by Davey et al. (2007) in which 23 different apple cultivars were inoculated with *Botrytis cinerea*; they observed that susceptibility to infection generally decreased as the commercial harvest date increased. These differences could be due to the different pathosystem analysed and the fact that the susceptibilities of immature apples were not assessed.

Quality parameters, such as acidity and maturity index, displayed significant differences between harvests. Differences in susceptibility could be related to senescence processes that occur during maturity but that were not directly correlated with the quality parameters studied.

At 4 °C in the compatible interaction (*P. digitatum*-oranges), no significant differences were found in growth rate between harvest dates at the 10^7 conidia mL^{-1} inoculum concentration. Differences between the greenest harvest and the other harvests were more pronounced at lower inoculum concentrations. Different results

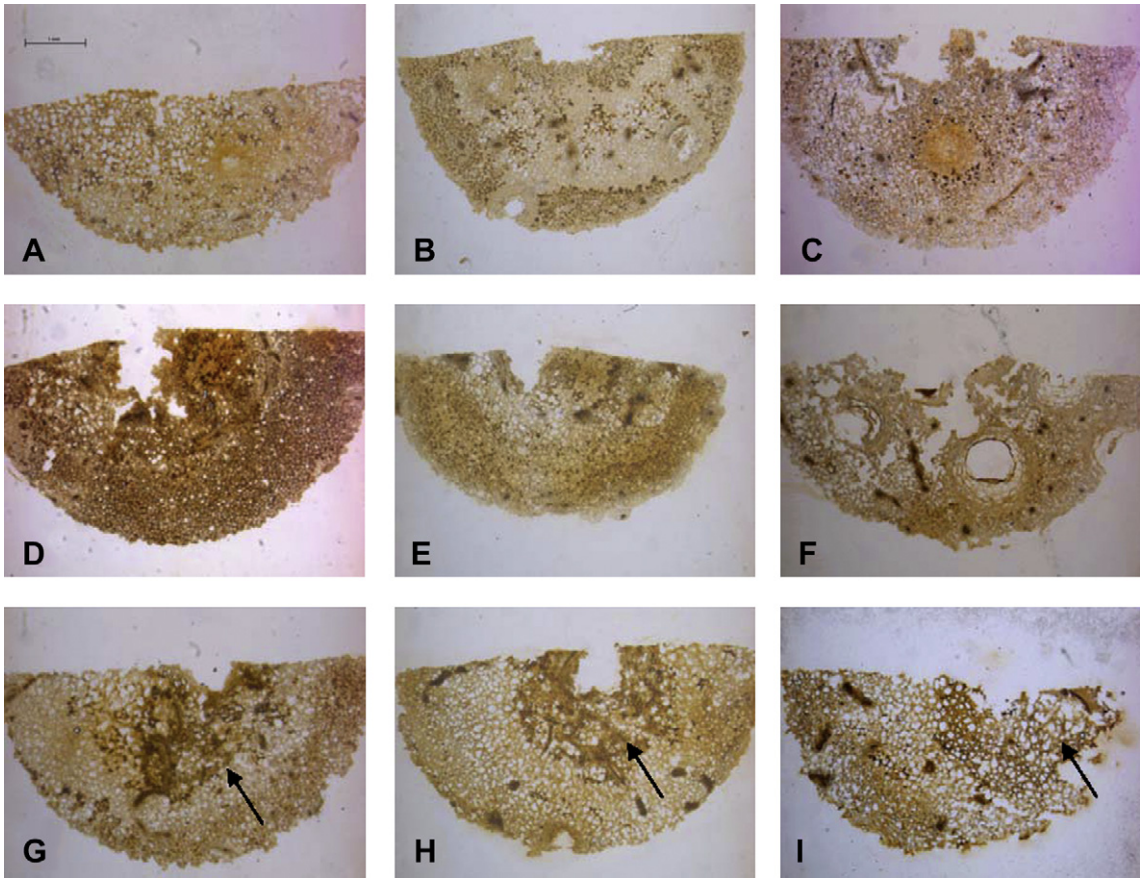

Fig. 7. Maüle tests for lignin in control (A to C), *Penicillium expansum* at the 10^4 conidia mL^{-1} concentration (D to F) and *P. expansum* at the 10^7 conidia mL^{-1} concentration (G to I) 7 d after inoculation and in immature (A, D and G), mature (B, E and H) and over-matured (C, F and I) oranges. Maüle tests resulted in typical orange-reddish-brown stains around the wounds, which is a positive sign for the presence of lignin compounds. Stereoscope magnification: 20x Scale bar = 1 mm.

were obtained by Boonyakiat et al. (1987) in pears inoculated with *P. expansum* at 10^4 conidia mL^{-1}; that study demonstrated that decay of inoculated fruit was higher in over-matured fruit, and no differences were found in the percentage of decay between immature and mature harvests.

Visible symptoms of decay appeared later at cold temperatures than at 20 °C. *P. digitatum*-related disease developed more slowly below 10 °C, and decay usually did not develop beyond the blister stage if fruits were stored at 1 °C (Eckert and Brown, 1986). In *in vitro* studies at 25 °C, *P. digitatum* achieved 100% spore germination within 15 h of inoculation, and germination was both delayed and slowed when temperature decreased to 10 °C and 4 °C (Plaza et al., 2003). In a *P. expansum*-apples interaction, Baert et al. (2007) demonstrated that shortened lag phases and growth rates were found when the temperature increased from 2 °C to 25 °C.

At both temperatures assayed, no differences were found in growth rate between inoculum concentrations at any harvest dates. Similar results were obtained by Baert et al. (2008), who found that inoculum concentration did not produce a clear effect on growth rate in apples inoculated with two different *P. expansum* strains at 25 °C and 4 °C. Morales et al. (2008) also reported no significant differences in growth rate between inoculum concentrations in apples inoculated with *P. expansum*, but only at a cold storage temperature.

To our knowledge, this is the first work that reports the capacity of *P. expansum* (non-host pathogen) to infect oranges under specific conditions. Our results at 20 °C indicated that when the concentration of this non-host pathogen decreased to 10^6 conidia mL^{-1}, infection could only develop in over-matured oranges. Meanwhile, at lower concentrations such as 10^5 or 10^4 conidia mL^{-1}, *P. expansum* was not able to induce decay. Previous studies (Baert et al., 2008) carried out in apples inoculated with *P. expansum* (compatible pathogen) showed that a minimum inoculum concentration is necessary to infect the fruit. The disease triangle illustrates that the existence of a disease caused by a biotic agent absolutely requires the interaction of a susceptible host, a virulent pathogen, and an environment favourable for disease development (Agrios, 2005). Thus, several biochemical changes could occur between immature and mature fruits to disrupt host defence and make oranges susceptible to non-host pathogens.

Macarisin et al. (2007) showed the capacity of *P. expansum* to germinate and temporarily grow in citrus fruits. However, they only observed infection in a citrus-*P. expansum* interaction when the wound was pre-treated with citric, ascorbic and oxalic acids (in all cases, a minimum concentration of 100 mM organic acid was necessary) and enzyme catalase (minimum concentration of 100 U mL^{-1}), suggesting that these substances help to suppress H_2O_2 production in the wound site. In contrast, we have demonstrated the ability of a non-host pathogen to infect oranges directly.

In the *P. expansum*-oranges interaction, linear growth patterns were observed between both temperatures assayed. In contrast to results obtained in the compatible interaction, decay incidence and severity were higher at 4 °C than at 20 °C. This behaviour could be explained by the possibility that at 4 °C, fruit wound-healing processes and defence mechanisms are slower than at room temperature, and some might even be inhibited. In studies performed in Valencia oranges, Ismail and Brown (1975) found that the healing rate at 5 °C was much slower than at 30 °C. Brown and Barmore (1983) showed that phenols and lignin-like materials appear responsible for resistance to infection in curing oranges. Mulas et al. (1996) showed that lignin biosynthesis is active at 20 °C and decreases at 2.5 °C. Moreover, *P. expansum* is mainly a cold storage condition pathogen (a “packinghouses” pathogen); therefore, it is well adapted to cold temperatures and could take advantage of this situation. Gougouli and Koutsoumanis (2010) reported that the lowest storage temperature at which *P. expansum* grew was −1.3 °C. At this temperature, a very slow increase of the mycelium diameter was observed after an extensive lag period of about one month.

A reaction in the tissue around wounds was clearly observed when oranges were inoculated with *P. expansum*, and this reaction increased proportionally to pathogen concentration and decreased as maturity advanced. To identify the possible compounds involved in this reaction, histochemical studies were performed. In the short-period response (48 h), the Maüle and toluidine blue O tests showed positive reactions for lignin in immature oranges in both compatible and incompatible interactions. This result suggests that the production of lignin-like substances is not exclusive for citrus pathogens. However, in control samples, the lignin reactions were always negative. For wounded pear tissue, Spotts et al. (1998) found a rapid accumulation of callose, tannins, and gum, but different tests for lignin were negative. In curing studies in lemons, Baudoin and Eckert (1985) found that the formation of these substances required wounding, and their abundance was increased if a pathogen or other elicitor was present.

Orange wounds inoculated with the non-host pathogen were analysed in the long-period response (7 d), and the lignin reaction over this time was more intense than that at 48 h. Lignification was apparently more important in immature fruits than in commercial mature fruits, and lignin-like material was not observed in over-matured fruits. However, Baudoin and Eckert (1985) showed that susceptible lemons (turgid or mature) produced lignin-like material more rapidly than more resistant/sub-turgid or less mature fruits after 5 days at 25 °C and 100% RH. These differences may be because these authors assessed a compatible interaction between lemons and *Geotrichum candidum*.

The toluidine blue O stain was also important for detecting microscopic rot development in the non-compatible interaction. In over-matured fruits, *P. expansum* conidia germination was found at the 10^4 conidia mL^{-1} inoculum concentration, but no visible rot symptoms were observed at this concentration.

No reactions were observed with histochemical tests used to detect suberin and callose in our samples. On the contrary, Lai et al. (2003) showed in NMR analysis that the peaks and signals obtained from grapefruit after wounding and fungal inoculation were comparable to those obtained from the suberised sweet potato epiderm. These results support the hypothesis that suberin is formed in the peel of grapefruit and excludes lignin as a possible component in the newly formed material.

In albedo tissue, a hypersensitive response (HR) was observed when *P. expansum* was unable to infect oranges. Macarisin et al. (2007) found that in citrus fruits, approximately 4–5 days after inoculation with *P. expansum*, the first indications of an HR became visible as evidenced by a front of dead, lignified cells on the edges of the wounds. The HR includes localised tissue collapse and cell death at the infection site. A visible necrosis occurs on the host tissue with a necrotic region, which arises as a result of very complex events in the host and constitutes a defensive barrier. While this barrier prevents the spread of biotrophic fungi (Lamb and Dixon, 1997; Lu and Higgins, 1999), it does not prevent the subsequent spread of necrotrophic fungi (Mayer et al., 2001) and in some cases could stimulate growth development (Govrin and Levine, 2000). However, peroxidases play a key role at a later stage in the phenylpropanoid pathway during the synthesis of lignin, which acts as a cell wall reinforcement enhancing resistance against pathogens (Ballester et al., 2010). Accordingly, both *P. expansum* and *P. digitatum* are necrotrophic pathogens, but the evident visual change was only observed in the incompatible interaction as a brownish and necrotic tissue near the infection site. This reaction could be attributed to HR and may be intended either

to retain pathogen growth (Glazebrook, 2005) or to form new physical barriers.

This research demonstrates that maturity stage could affect the infection capacity in compatible and incompatible interactions in oranges. Additionally, lignin formation in combination with death tissue may play an important role in the defence mechanisms against *P. expansum*. Until now, *P. expansum* was considered as non-host pathogen of oranges, but in this study, it has been demonstrated that from the commercial harvest, an incompatible interaction can become compatible if favourable conditions present themselves.

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